



Engineered FnCas9 mediated mutation profiling for clarithromycin resistance in *Helicobacter pylori* strains isolated from Indian patients with gastrointestinal disorders

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ARTICLE INFO

Keywords:

23S ribosomal DNA
Antibiotic resistance
CRISPR-Cas9
FELUDA
Gastrointestinal diseases
Helicobacter pylori

ABSTRACT

Helicobacter pylori is a highly prevalent gut pathogen with reported implications in a wide range of gastrointestinal disorders. Antibiotic based therapy, especially with clarithromycin is one of most effective treatment strategies against *H. pylori*. However, rising global prevalence of clarithromycin resistance in certain *H. pylori* strains, primarily attributed to point mutations in the 23S ribosomal RNA coding gene, pose a major challenge in effective eradication of this pathogen. There are a number of established methodologies devised for *H. pylori* mutation detection, so as to provide a tailored treatment plan to the patients and resist further transmissions of antibiotic resistant strains. However, there is no 'gold standard' method available to detect mutation status in clinical isolates of *H. pylori* from infected patients. CRISPR-Cas9 based technologies have revolutionized the field of mutation detection in biological samples, particularly during the recent COVID-19 pandemic. Although multiple assays have been reported for detection of *H. pylori* in clinical samples including CRISPR diagnostics (CRISPRDx) platforms, there is no such assay reported till date to detect specific mutations that confer antibiotic resistance to this pathogen. In this study, we have developed an assay using engineered FnCas9 (en31-FnCas9) protein to effectively detect the A2142G and A2143G mutations in the 23S rDNA of *H. pylori* strains isolated from gastric biopsy samples. The data from *in vitro* cleavage assays and strip-based lateral flow tests using en31-FnCas9 and guide RNAs targeting the conserved and mutated loci of *H. pylori* 23S rDNA are in perfect congruence with the data from Sanger sequencing and restriction fragment length polymorphism (RFLP) analysis. Our results indicate that en31-FnCas9 based mutation analysis can be deployed as an efficient diagnostic methodology to detect clarithromycin susceptibility in patients with suspected *H. pylori* infections.

1. Background

Helicobacter pylori is a gram-negative pathogenic bacterium that colonizes human stomach. Infections with *H. pylori* are quite common, affecting over 43 percent of the world's population [1]. Presence of this gut pathogen in human is linked to a wide range of gastrointestinal

disorders, including peptic ulcers, gastritis, dyspepsia and even gastric cancer [1]. Early detection of this gastric pathogen can be instrumental in providing effective course of treatment along with prevention of further transmissions. While antibiotics including clarithromycin, metronidazole, amoxicillin and bismuth compounds are usually the first line of therapeutics for patients with *H. pylori* infections, emergence of

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antibiotic resistance has become a challenge in successful eradication of this pathogen [2]. Especially the resistance of *H. pylori* to clarithromycin has now become a global therapeutic challenge, having a world-wide prevalence of 27 % with regional variation as suggested by a recent systematic review [3]. The most explored mutations of clarithromycin resistance are A2143G and A2142C/G single nucleotide variations located at 23S rRNA coding gene, which alters the ribosomal binding site for this antibiotic compound [2]. Rapid identification of not only *H. pylori* but also the nature of antibiotic resistance will allow physicians to choose right antibiotics for its eradication [4]. Therefore, integration of novel diagnostic strategies as cost-effective tools to detect the presence of *H. pylori* in human samples, as well as the identification of the antibiotic susceptibility is crucial for its rapid eradication.

A number of PCR-based experimental methodologies including ARMS-PCR, nested PCR and PCR-based denaturing HPLC have been devised for molecular diagnosis of the mutations implicated with clarithromycin resistance in *H. pylori* strains. [5–8]. Despite being sensitive and specific for certain mutation sites, execution of the aforementioned techniques can be difficult while detecting different mutations in a wide variety of samples [9]. Multiplex real-time PCR and Taqman-based assays have emerged as effective strategies to detect the presence of *H. pylori* in patients [10,11]. However, heterogeneity of clinical samples might lead to limited efficiency in real-time detection of specific mutations implicated in the antibiotic resistance [12]. Methods involving restriction fragment length polymorphism (RFLP) analysis to detect antibiotic resistance mutations is an effective approach, but it might not be the most suitable methodology to deliver quick diagnostic reports [13]. Sanger sequencing or next generation genome sequencing have unparalleled accuracy for mutation detection, however the widespread adoption of sequencing for diagnostic purposes is challenging owing to its association with higher experimental cost and turnaround time [14,15]. Therefore, it is important to develop alternate mutation detection techniques in *H. pylori* infected patients, so as to foster advancement in precision medicine guided by the antibiotic resistance pattern of this gastric pathogen.

The CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated proteins) system has emerged as an innovative tool to detect and manipulate genomic loci in a wide range of sample genomes with high precision and versatility [16–18]. A combination of various PCR techniques along with CRISPR-Cas12a based detection methodologies have been successfully used by a number of research groups across the globe to detect *H. pylori* in patient-derived samples [19–24]. CRISPR-Cas13a based assays are also shown to be effective in rapid identification of *H. pylori* in clinical isolates [25]. Therefore, CRISPR can offer thorough understanding of *H. pylori* genetics by identification of different genes linked to antibiotic resistance, virulence and other important parameters [26]. In the realm of identifying the genetic mutations implicated in antibiotic resistance of *H. pylori*, CRISPR-based methodologies are known to enable site recognition and cleavage of the target DNA with exceptional accuracy by designing guide RNAs targeting the respective mutation site in various kinds of DNA samples [17,18]. An in-depth understanding of *H. pylori* genetic makeup by CRISPR-based diagnostics (CRISPRDx) would therefore aid in molecular dissection of its pathogenicity and development of targeted therapeutics against different strains.

The well-recognized biosensing properties of CRISPRDx prompted us to evaluate its applicability for detection of *H. pylori* mutations implicated in clarithromycin resistance among patients. Towards this goal, our group had previously demonstrated the possibility of detecting *H. pylori* antibiotic resistance mutations using Cas9-based mutation detection strategies [27,28]. However, despite a number of advantages of CRISPRDx as described, CRISPR-Cas9 based biosensing techniques face limitations due to the requirement of NGG PAM sequences at the recognition site while detecting mutations [29,30]. To encounter this limitation of CRISPR-Cas9 based detection tools, we have recently developed an in-house version of engineered Cas9 protein having

resemblance to Cas9 orthologs isolated from *Francisella novicida* (en31-FnCas9) but with altered PAM binding affinity. In this study, we have reported the potential of en31-FnCas9 to successfully detect the presence and identify the 23S rDNA mutation status of *H. pylori* in gastric biopsy samples from dyspeptic patients, both by *in vitro* cleavage studies and lateral flow-based test strip assays (FELUDA) [31]. A schematic representation of common molecular diagnostic techniques for detection of *H. pylori* and its antibiotic resistance pattern in patient samples has been provided in Fig. 1. Our results from the en31-FnCas9 based detection methods were in perfect congruence with the mutation reports derived from Sanger sequencing and RFLP for the same samples. This is the first report of en31-FnCas9 mediated molecular diagnosis of *H. pylori* mutations implicated in clarithromycin resistance.

2. Results

2.1. Prevalence of *H. pylori* mutations among the study participants

Ninety patients with dyspepsia and peptic ulcer disease were recruited from All India Institute of Medical Sciences (AIIMS) New Delhi and AIIMS Bhubaneswar. Details on the clinical observations of the study participants have been provided in Table 1. *H. pylori* DNA was isolated as per the protocol described in methods section. Forty-four patients were found to be *H. pylori* positive, as evidenced by PCR product (166 bp) formed against the 23S rDNA gene of this gastric pathogen (Table 1). PCR amplified products from the 44 *H. pylori* positive samples were purified with column-based technique before sending for Sanger sequencing, as mentioned in the methods section. After the sequencing of *H. pylori* 23S rDNA from gastric biopsies, 41 samples (93.2 %) were found to be wild type (AA) for the target loci. Among the mutations confirmed by sequencing, two of the *H. pylori* clinical isolates (4.5 %) were positive for both A2142G and A2143G mutations (GG1 and GG2) and one sample (2.3 %) was found to be positive for only A2142G mutation (GA) (Fig. 2a,b). None of the sequenced samples harboured the A2142C mutation (Fig. 2a).

2.2. *H. pylori* mutation detection by RFLP methodology

BsaI and BbsI restriction endonucleases are widely used in detection of *H. pylori* A2143G and A2142G mutations, respectively [13]. To assess the efficiency of RFLP in mutation detection, plasmid constructs of *H. pylori* 23S rDNA sequence harbouring wild type (AA), 2142G/2143G (GG) and 2142C/2143G (CG) variants were generated. Thereafter, the 23S rDNA target site (425 bp) in the plasmids were PCR amplified and subsequently digested with BsaI according to the usual protocol. The digested products were run on a 2% agarose gel. A distinct difference could be observed in the restriction digestion pattern with the occurrence of A2143G mutation, as evident by no digestion in wild-type (AA) plasmid and two bands generated in both GG and CG plasmid constructs (Fig. 3a).

Furthermore, RFLP was done with the *H. pylori* DNA derived from gastric biopsy samples. Sequenced *H. pylori* DNA samples with confirmed A2142G (GA) and A2142G + A2143G mutations (GG1 and GG2), along with one of the wild-type samples (AA) were PCR amplified to generate 425 bp fragments as described earlier. Samples with A2142G + A2143G mutations (GG1 and GG2) were digested with BsaI enzyme along with wild type control and electrophoresed as described earlier. Both the samples with GG double mutations showed cleavage upon digestion with BsaI, whereas the wild type substrate remained intact (Fig. 3b). PCR products of DNA with A2142G mutation (GA) and wild type sample were digested with BbsI and electrophoresed in a 2% agarose gel, alongside undigested controls. A2142G mutation was confirmed by cleavage of PCR product only in the mutation-harbouring sample (GA) upon BbsI digestion (Fig. 3c).

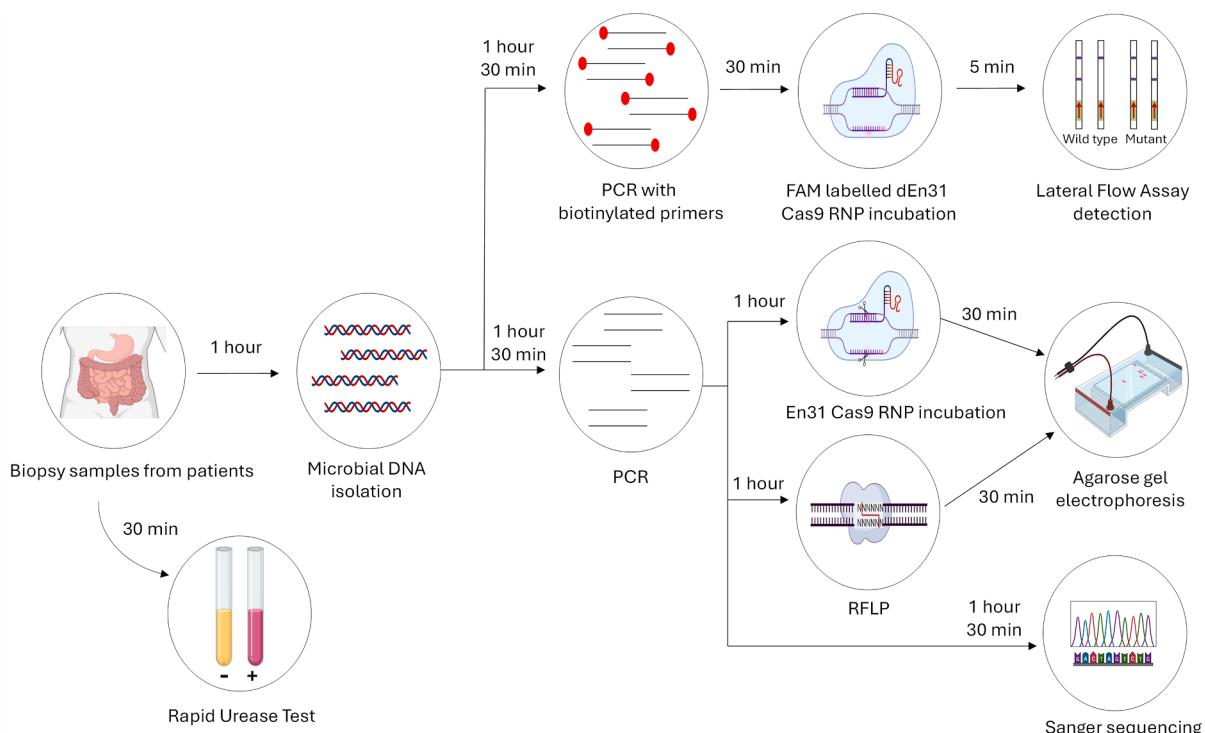


Fig. 1. Schematic representation of various diagnostic methods for *H. pylori* mutation detection and interpretation of the data.

Table 1
Clinical observations of the study participants.

Parameters	Patient samples [n (%)]	
	<i>H. pylori</i> positive [n = 44]	<i>H. pylori</i> negative [n = 46]
1. Diagnosis		
Functional Dyspepsia	Functional Dyspepsia 40 (90.9)	Functional Dyspepsia 39 (84.8)
• EPS- 14 (35)	• EPS- 19 (48.7)	
• PPDS- 15 (37.5)	• PPDS- 15 (38.5)	
• EPS + PPDS- 11 (27.5)	• EPS + PPDS- 05 (12.8)	
Peptic Ulcer Disease	Peptic Ulcer Disease 04 (9.1)	Peptic Ulcer Disease 07 (15.2)
2. Age (years) [Median (min, max)]	42.5 (27–70)	40 (21–68)
3. Gender		
Female	12 (27.3)	21 (45.7)
4. Duration of Symptoms (Median, in years)	02	02
5. UGIE reports		
Antral erythema	31 (70.5)	05 (10.9)
Antral/duodenal ulcer	07 (15.9)	01 (2.2)
Corpoantral erythema	04 (9.1)	09 (19.6)
Normal study	00 (0)	25 (54.3)
Other	02 (4.5)	06 (13)
6. <i>H. pylori</i> detection¹		
RUT only	06 (13.6)	10 (21.7)
Bx only	24 (54.6)	16 (34.8)
RUT + Bx	14 (31.8)	20 (43.5)
7. Histopathological observations		
Chronic active gastritis	40 (91)	10 (21.8)
Chronic pangastritis with metaplasia	02 (4.5)	03 (6.5)
Mild/moderate gastritis	02 (4.5)	33 (71.7)

n = sample number in the respective category; EPS = Epigastric pain; PPDS = Postprandial distress syndrome (PDS); UGIE = Upper gastrointestinal endoscopy; RUT = Rapid Urease Test; Bx- Biopsy Report.

¹ RUT was performed in a subset of 50 study participants.

2.3. Detection of *H. pylori* and analysis of mutations by en31-FnCas9

To detect the applicability of en31-FnCas9 protein in detecting *H. pylori* and identifying the clarithromycin susceptibility in the same sample, two different sgRNAs were designed against the *H. pylori* 23S rDNA sequence. The sgRNA detecting presence of *H. pylori* in patients was designed to target the conserved region of 23S rRNA (sgcons) that do not harbour any mutation. Whereas, mutation status of the *H. pylori* clinical isolates was planned to be detected by another sgRNA targeting the mutation-harbouring site of the 23S rDNA (sgdiff) (Fig. 4a). Owing to the PAM flexibility of en31-FnCas9, the sgdiff was designed by the altered PAM strategy, where both A2142G and A2143G mutations could serve as the PAM recognition site for the enzyme. As a result, only the mutated locus in the 23S rDNA sequence shall be targeted by en31-FnCas9, and the wild-type locus shall be left unperturbed due to PAM non-availability. Detailed description regarding the sequence of genomic target and both sgRNAs has been provided in Fig. 4b.

Both the sgRNAs were prepared and purified according to the procedure described in the methods section. PCR amplified 23S rDNA products (425 bp) of all three *H. pylori* clinical isolates with confirmed mutations (GG1, GG2 and GA) and one wild type sample were taken into consideration. *In vitro* cleavage (IVC) assay was performed with the purified PCR products and en31-FnCas9 ribonucleoprotein complexes (RNP) for both sgRNAs (sgcons and sgdiff), according to the protocol mentioned in the methods section. Upon visualization of the IVC products on agarose gel, it was observed that sgcons could successfully cleave the PCR products for all the samples, irrespective of their mutation status. Whereas sgdiff was found to cleave only the mutated target loci, leaving the wild type locus intact (Fig. 4c). The results indicate that our strategy was successful in exploiting the PAM flexibility of en31-FnCas9 to detect *H. pylori* 23S rDNA mutations conferring clarithromycin resistance in patients.

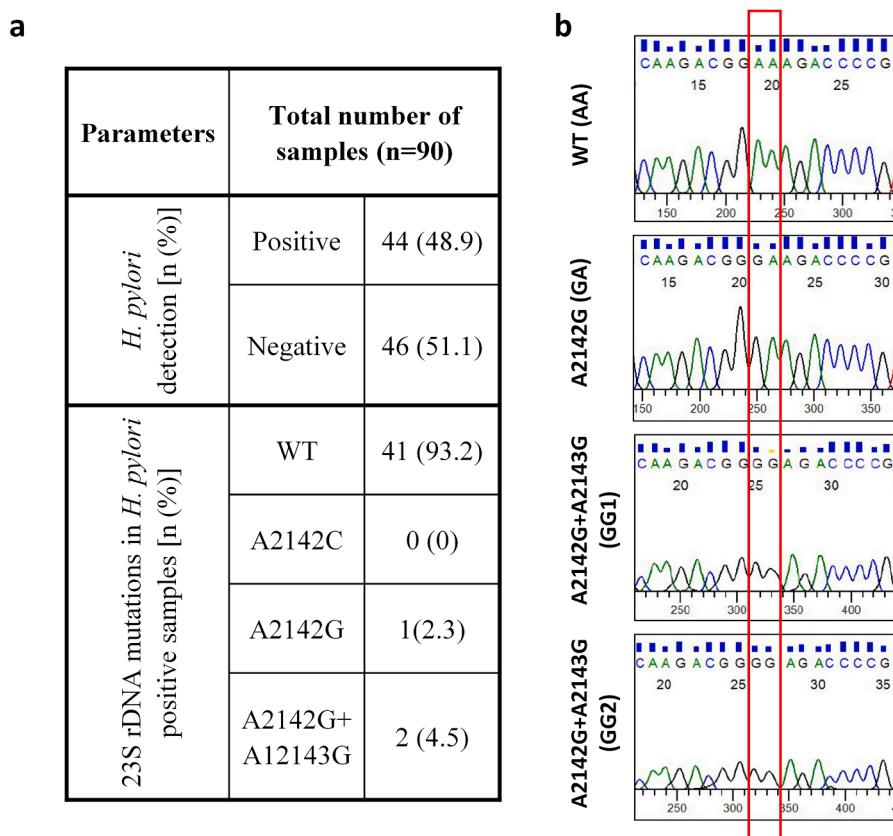


Fig. 2. Mutation analysis of *H. pylori* strains isolated from gastric biopsy samples. **a** Table indicating the percentage of *H. pylori* positive samples and the frequency of 23S rDNA mutations conferring clarithromycin resistance in the same, as confirmed by sanger sequencing. **b** Sequencing data of the 23S rDNA target loci for one wild type (AA) sample and all the samples with confirmed mutations (GA, GG1 and GG2).

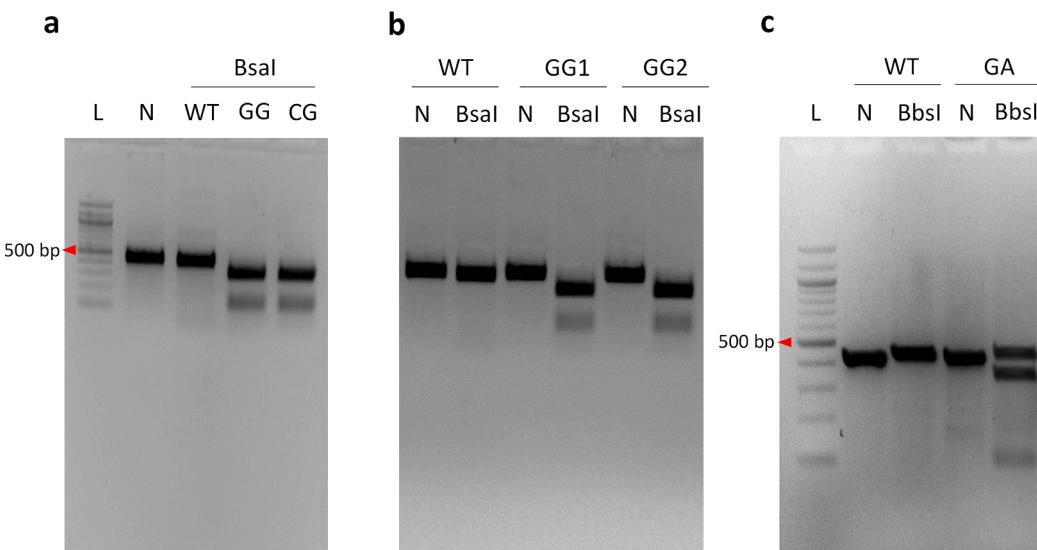


Fig. 3. RFLP data for *H. pylori* 23S rDNA sequences from synthetic plasmid constructs and clinical isolates from patients. Representative RFLP analysis outcomes on BsaI restriction enzyme digestion of PCR amplified 23S rDNA sequence (425 bp) from **a** *H. pylori* plasmid constructs harbouring wild type (WT), A2142G + A2143G (GG) and A2142C + A2143G (CG) mutations and **b** patient-derived *H. pylori* strains with wild type (WT) and A2142G + A2143G (GG1 and GG2) mutations. Here 'N' represents the PCR amplified products without restriction digestion and 'BsaI' represents the amplified products after BsaI digestion, which on cleavage produces two product sizes of 325 bp and 100 bp respectively. **c** Representative RFLP analysis outcomes on BbsI enzyme digestion of 23S rDNA PCR products (425 bp) from *H. pylori* clinical isolates with wild type (WT) and A2142G mutations (GA). Here 'N' represents the amplified product without restriction digestion and 'BbsI' represents the amplified products after BbsI digestion which on cleavage produces two products again (325 bp and 100 bp).

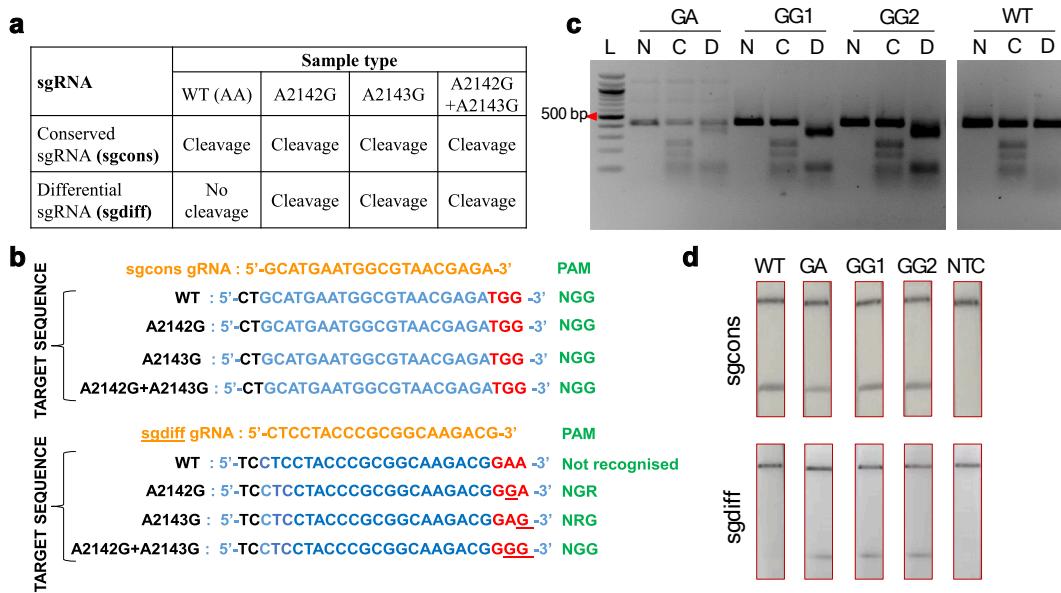


Fig. 4. Detection of *H. pylori* and 23S rDNA mutations conferring clarithromycin resistance by en31-FnCas9 based strategy. **a** Table depicting the cleavage pattern expected from different samples when using both the conserved sgRNA (sgcons) and differential sgRNA (sgdiff). **b** Strategy behind the activity of sgcons and sgdiff in detection of the *H. pylori* 23S rDNA conserved region and mutations, respectively. The sgRNAs are shown in orange colour, corresponding target is in blue colour and the possible PAM is in red colour. For sgcons, the PAM sequence is always available at the chosen conserved site of 23S rDNA. In case of sgdiff, NGR, NRG or NGG type PAMs are formed due to the presence of the mutation itself, that are recognised by en31-FnCas9; whereas in case of wild type there is no en31-FnCas9 recognised PAM and there would be no cleavage. **c** Representative IVC outcomes on *H. pylori* wild type (WT), A2142G mutant (GA), A2142G + A2143G mutant (GG1 and GG2) substrates (425 bp) interrogated by en31-FnCas9 in the form of RNP complexes. 'N' represents the amplified product without RNP, 'C' represents the cleavage reaction with sgcons which on cleavage yields two product sizes (250 bp and 175 bp) and 'D' represents the cleavage reaction with sgdiff which on cleavage yields another two product lengths (320 bp and 105 bp). **d** Representative FELUDA for GA, GG1, GG2 and WT *H. pylori* samples with sgcons (upper lane) showing successful detection of all the *H. pylori* positive samples, and sgdiff (lower lane) showing successful mutation detection in GA, GG1 and GG2 samples, but no test band in the WT sample. NTC = no template control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.4. Establishment of lateral flow assay (FELUDA) for rapid detection of *H. pylori* and identification of clarithromycin resistance in patient samples

Lateral flow assays are a popular choice as the point-of-care diagnostic devices, owing to their ability to deliver quick results with a visual demonstration. In our study, we wanted to pair the en31-FnCas9 mediated *H. pylori* mutation detection strategy with a strip-based lateral flow assay, in order to extend its applicability as a diagnostic methodology in a clinical setup. As shown in the earlier report from our group, the identification of target loci onto the test strip can be achieved via detection of RNP-bound biotinylated products using FAM-labelled chimeric gRNA molecules, a methodology known as 'FELUDA' [27,28]. Briefly, 239 bp 23S rDNA biotinylated amplicons were generated with the *H. pylori* clinical isolates for all three mutated strains (GG1, GG2 and GA) and one of the wild type controls as confirmed by sequencing, RFLP and IVC assays. Two separate dead en31-FnCas9 RNP complexes were generated with each of the chimeric FAM-labelled gRNAs, i.e. one against the conserved region of *H. pylori* 23S rDNA (sgcons) and another targeted for the mutation detection in same (sgdiff). Thereafter, respective RNP complexes were incubated with the biotinylated amplicons before loading onto the FELUDA test strip. Upon experimentation with sgcons (Fig. 4d, upper lane), the test strips showed two prominent bands in both wild type and mutated (GA, GG1, GG2) samples (one for control, another for test), while there was only a single control band in the no-template control.

On the other hand, in case of sgdiff, only the mutated 23S rDNA locus was supposed to be targeted by en31-FnCas9, and the wild-type locus should be left unperturbed due to PAM non-availability. As per our hypothesis, upon performing FELUDA with sgdiff (Fig. 4d, lower lane), only the samples with mutated 23S rDNA loci were targeted by en31-FnCas9, giving rise to two bands for mutant samples- whereas single control bands were observed for both wild type sample and the no-

template control. Taken together, we report a rapid visual readout of *H. pylori* infection and detection of its clarithromycin resistance status using FELUDA assay in patient-derived samples, with the potential to be developed further for point-of-care applications.

3. Discussion

Mutations implicated in antibiotic resistance of *H. pylori* are found to be more prevalent in countries with higher rates of overuse and misuse of antibiotics [32]. The estimated global prevalence of clarithromycin-resistant mutations in *H. pylori* was previously reported to be around 27 percent [3,33]. In the present study, we have found that 6.7 % of the patients harboured mutations at the target site of 23S rRNA coding gene, suggesting clarithromycin resistance in these patient-derived *H. pylori* strains. Being one among the first-line of medications, resistance to clarithromycin in *H. pylori* may cause failure in the successful eradication of *H. pylori* infection in such patients. There are earlier reports supporting invasive molecular diagnostic techniques for higher reliability in *H. pylori* detection from patients under consideration [34]. Moreover, mutation status of *H. pylori* strains is not detectable by the most widely used non-invasive techniques to detect an existing infection, e.g. rapid urease test (RUT) and urea breath test (UBT). Therefore, an integration of molecular detection techniques for *H. pylori* and its antibiotic susceptibility profile from patient tissue samples is the most effective method for diagnosis of *H. pylori* infection. We have reported a novel strategy with effective use of en31-FnCas9 protein to recognize *H. pylori* and its mutation status regarding clarithromycin resistance in people with a suspected infection with this gastric pathogen. The perfect congruence of the en31-FnCas9 based results with that of Sanger sequencing and RFLP further affirms the applicability of this experimental design to detect *H. pylori* mutations in patient samples.

CRISPR-Cas based experimental strategies are proven to facilitate

effective detection of *H. pylori* from patients across the globe, as discussed earlier [19–26]. In this study, we have leveraged the unique binding proficiency of engineered en31-FnCas9, which exhibits recognition capabilities for NGA PAM sequences [31]. The versatility of this particular CRISPR-based system, owing to its PAM flexibility, allows for accurate mutation identification in a wide range of genomic sequences irrespective of the sample identity. While it is difficult to absolutely eliminate the chances of manual errors when handling PCR amplification products on membrane-based lateral flow assay strips, we have taken extensive precautions to minimize contamination risks. This includes clearly separating pre- and post-amplification areas and designating specific storage spaces for all reagents, kits, test strips, and plasticware used throughout the experiment. Our study marks the inaugural documentation for the potential of en31-FnCas9 to detect A2143G and A2142G mutations in clinical isolates of *H. pylori* derived from patient gut biopsies, both by *in vitro* cleavage studies and test-strip based FELUDA assays. Moreover, further explorations on this novel strategy can lead to successful implementation of the same for antibiotic resistance mutation detection in *H. pylori* strains isolated from a wide range of patient-derived biological samples, including stool, blood, saliva and dental plaques. Combination of this en31-FnCas9 based bio-sensing strategy with membrane-based lateral flow assay points towards the possibility of extending its applicability as a commercial molecular diagnostic procedure with a visual output to detect various antibiotic-resistant mutations in this gastric pathogen. Future direction of the present study shall be aimed at evaluation of various clinical, anthropometric and biochemical parameters in a larger study population comprised of dyspeptic patients, so as to gain a thorough insight on various risk factors implicated in *H. pylori* infection and attainment of antibiotic resistance in the same.

Antibiotic resistance in *H. pylori* poses a global threat to public health by necessitating repeated diagnostic tests and use of multiple courses of different antibiotic combinations for eradication of the same. Some of the antibiotic-resistant mutations in *H. pylori* are indirectly associated with risks of gastric cancer in patients [35]. Effective molecular diagnosis of *H. pylori* mutations may prove to be instrumental in order to provide a tailored treatment plan and mitigate the spread of antibiotic-resistant strains. The present study provides a novel strategy not only to detect the presence of *H. pylori* but it also identifies the mutation status simultaneously in the biopsy sample. Our future goal is to advance this technology as an affordable point-of-care diagnostic tool for detecting *H. pylori* and its mutations in dyspeptic patients from rural areas in India, where access to diagnostic laboratories is limited or non-existent. By

drastically reducing sample processing costs and experimental time, as well as eliminating the need for advanced scientific equipment like real-time PCR machines, this method offers the potential to provide timely results even in remote villages. The rapid and precise identification of antibiotic resistance patterns with this technique would enable clinicians to make informed treatment decisions and help to curb the spread of antibiotic-resistant strains. Successful deployment of this methodology in any clinical setup would therefore broaden the avenue for effective management of this global public health concern.

4. Methods

4.1. Oligos

Sequence of all the oligos used in the study are mentioned in Table 2.

4.2. Plasmid construction

Constructs of *H. pylori* 23S rDNA sequence (837 bp) bearing wild type (AA), 2142G/2143G (GG) and 2142C/2143G (CG) variants were synthesized and cloned into pUC57 vector backbones (GenScript Biotech Pvt. Ltd.). The synthesized plasmid constructs were sequenced via Sanger technology to confirm the presence of the *H. pylori* sequences cloned with mutations at the target site.

4.3. Procurement of patient samples

A total of 90 patients admitted at the AllMS Bhubaneswar and AIIMS New Delhi between the time period of 2018 to 2022 with suspected *H. pylori* infections were included in the study. Tissues for biopsy were procured from both gastric body and antrum during upper gastrointestinal tract endoscopy of the patients. Rapid urease test (RUT) was performed in a subset of 50 patients. Tissue samples were immediately immersed in RNAlater solution (Thermo Fischer Scientific Inc.) and stored at –80 °C until further processing.

4.4. Isolation of *H. pylori* genomic DNA from tissue biopsies

Patient tissue samples were brought to room temperature and lysed via rigorous vortexing with a bead beater homogenizer (Scientific Industries Inc.). *H. pylori* DNA from the lysed tissue samples were isolated by the DNeasy 96 PowerSoil Pro QIAcube HT Kit (Qiagen India Pvt. Ltd.) according to the manufacturer's instructions. Concentration and purity

Table 2
List of oligos used for the study.

Experiment	Oligo name	Sequence (5' to 3')	Product size (bp)
Sanger Sequencing	HP_Seq_F HP_Seq_R	AGATGGGAGCTGTCTCAACC AAAGCCCTTACTTCAAAGGCC	166
IVC/RFLP amplicon PCR	HP_RE_F HP_RE_R	CCACAGCGATGTGGTCTCAG CTCCATAAGAGCCAAAGCCC	425
FELUDA amplicon PCR	HP_biotin_F HP_biotin_R	(Biotin)GGTCCTAAGGTAGCGAAATTCC (Biotin)CTCCATAAGAGCCAAAGCCC	239
IVC gRNA	sgcons_F sgdiff_F FnCas9_universal_R	TAATACGACTCACTATAGCATGAATGGCGTAACGAGAGTTTCAGTTGCCTGAAAGGCCTCTGTAATCATT TAATACGACTCACTATAGCTCTACCCGGCAAGACGGTTTCAGTTGCCAAAGGGCTCTGTAATCATT CAGACGTGCAAACAGAGGTCCGTTAAATACTTTAAATGATTACAGAGCGCTTCGGCGCAACTGAAAC	
FELUDA gRNA	sgcons_F sgcons_R sgdiff_F sgdiff_R tracrRNA_FnCas9_FAM	TAATACGACTCACTATAGCATGAATGGCGTAACGAGAGTTTCAGTTGCCTGAATTAT ATAATTTCAGCAACTGAAACTCTCGTTACGCCATTCTAGCTATAGTGAGTCGTATTA TAATACGACTCACTATAGCTCTACCCGGCAAGACGGTTTCAGTTGCTGAATTAT ATAATTTCAGCAACTGAAACCGCTTGCCTGGTAGGAGGCTATAGTGAGTCGTATTA G*U*AAUUAUUGUCUGUAUAAAAGUAUUUGAACGGACCUUGACACGU*C*U-FAM	

of the DNA samples were estimated using a NanoDropTM 2000 spectrophotometer machine (Thermo Fischer Scientific Inc.).

4.5. Sanger sequencing

A total of 44 *H. Pylori* DNA samples isolated from patient biopsies were PCR amplified to generate a 166 bp amplicon using respective primer pairs as mentioned in Table 2. The amplified fragments were purified using column-based PCR product purification methods (Thermo Fischer Scientific Inc.), according to the manufacturer's protocol. Purified PCR products were run on a 2% agarose gel for size confirmation and sequenced via Sanger technology. Obtained results were read using the in-built software of the sequencer machine (Applied Biosystems corp.).

4.6. RFLP

For RFLP with *H. pylori* 23S rDNA plasmid constructs harbouring wild-type, GG and CG mutations, the target sites were PCR amplified to generate 425 bp amplicons of each using designated primer pairs as mentioned in Table 2. The PCR products were digested with BsaI restriction enzyme (New England Biolabs, Inc.) targeting 2143G mutation, as per the manufacturer's instructions. Digested plasmids were electrophoresed in a 2 % agarose gel along with one undigested control sample.

For RFLP with patient samples, the 23S rDNA sequence harbouring the mutation site in *H. pylori* clinical isolates were PCR amplified (425 bp) as described earlier. Thereafter the PCR products were digested either with BbsI or BsaI restriction enzymes. Restriction digested fragments were run via gel electrophoresis in a 2% agarose gel, along with undigested PCR products.

4.7. Design and preparation of crRNAs and sgRNAs

Preparation of sgRNAs and crRNAs was done by *in vitro* transcription using MegaScript T7 Transcription kit (ThermoFisher Scientific), as described earlier [27,28]. sgRNAs for *in vitro* cleavage (IVC) assays were prepared by anneal-extension PCR with the respective forward primers and the FnCas9 universal reverse primer (Table 2), as described before [27,28]. All crRNAs were purified by NucAway spin column (ThermoFisher Scientific Inc.). Chimeric gRNAs (crRNA:TracrRNA) for FELUDA were prepared via combining respective crRNAs and synthetic 3'-FAM-labeled TracrRNA (crRNA:TracrRNA molar ratio, 1:1) in an annealing buffer (100 mM NaCl, 50 mM Tris-HCl pH8 and 1 mM MgCl2) by heating at 95°C for 2–5 min and then allowing to cool at RT for 15–20 min, as described earlier [27,28].

4.8. IVC assay

DNA from *H. pylori* clinical isolates were PCR amplified using primer pairs against the 23S rDNA amplicon (425 bp) and used as substrate in IVC assays. Purified target amplicons were incubated in reaction buffer (20 mM HEPES, pH 7.5, 150 mM KCl, 1 mM DTT, 10 % glycerol, 10 mM MgCl2) along with reconstituted en31-FnCas9 RNP complex (500 nM) at 37°C for 30 min, as optimized in our previous study [31]. IVC products were electrophoresed on a 2% agarose gel and the results were observed.

4.9. FELUDA with gRNAs against the conserved and mutation site of *H. pylori* 23S rDNA

A 239 bp amplicon containing the mutation-harbouring or wild type locus was generated using a double end 5' biotin-labelled primer pairs, for each sample under consideration. Dead en31-FnCas9 RNPs were prepared by equally mixing (Protein:sgRNA molar ratio, 1:1) and chimeric gRNA (sgcons or sgdiff) in a buffer (20 mM HEPES, pH 7.5, 150 mM KCl, 1 mM DTT, 10 % glycerol, 10 mM MgCl2), as described earlier

[31]. RNPs were incubated for 10 min at RT prior to use. Biotinylated amplicons were incubated with the RNP complex in equal volumes (10 µL) for 10 min at 37°C. Finally, required volume of Dipstick buffer was added to the reaction along with the lateral flow strip (Milenia Biotech GmbH). The strips were allowed to stand in the solution for 2 min at RT and results were observed visually.

5. Ethics approval and consent to participate

Our study was approved by the Human Ethics Committees of All India Institute of Medical Sciences (AIIMS) New Delhi, AIIMS Bhubaneswar and CSIR-Institute of Genomics and Integrative Biology, New Delhi. The study was performed in accordance to the principles of Helsinki declaration of 1975 as revised in 1983. All study participants were pre-informed about the collection of tissue biopsy samples and written consents were obtained.

6. Consent for publication

All the authors and the affiliated institutes gave their consent for the publication.

CRediT authorship contribution statement

Shraddha Chakraborty: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Shubham Rana:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Sneha Gulati:** Methodology, Formal analysis, Data curation. **Shubham Chaudhary:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Manas K. Panigrahi:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Vinay K. Hallur:** Writing – review & editing, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **Souvik Maiti:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Debojyoti Chakraborty:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Govind K. Makharla:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Conceptualization.

Funding

Major funding for the study was provided by the Council of Scientific and Industrial Research, Government of India [MLP2009]. S.C. thanks Department of Science & Technology, Government of India for the DST-INSPIRE faculty fellowship.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Authors thank all the participants for donating their tissue biopsy samples for the study. Some editable figure icons used in the schematics were sourced from BioRender (www.biorender.com).

Data availability

No data was used for the research described in the article.

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